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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

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Full Length Research Paper

The epidemiology of malaria among pregnant women in Garoua, Northern Cameroon

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Epidemiological studies of malaria in pregnant women who live in Garoua, malaria endemic areas are scarce. The present study was undertaken to investigate the prevalence and distribution of malaria in the area. A total of two hundred and forty (240) pregnant women participated in the survey from January to March, 2007. Out of the 240 pregnant women examined, 97 were found to have malarial infections during the study, with *Plasmodium falciparum*. There was significant difference between prevalence of the infection at different stages of pregnancy (χ^2 = 0.088, df = 2, P < 0.05). Further investigations showed that 101 (44.44%) of the pregnant women had no bed nets for various reasons. Implication of malaria in pregnancy cannot be over emphasized. The need to vigorously pursue acceptance of control methods like the bed nets are discussed.

Key words: Malaria, prevalence, pregnancy, women.

INTRODUCTION

Malaria is endemic throughout most of the tropics. Of the approximately three billion people living in 108 countries who are exposed, approximately 243 million will develop symptomatic malaria annually World Health organisation (WHO, 2008). Malaria deaths peaked at 182 million in 2004 and felt to 124 million in 2010. Over 80% of the deaths occur in Sub-Saharan Africa (WHO, 2011).

Malaria during pregnancy is a major public health problem; it increases the risk of low birth weight, infant mortality and morbidity during the first year of life by inducing intra-uterine growth retardation, prematurity, and infant anaemia (Nosten et al., 1994). In malaria-endemic countries, placental malaria is associated with a 2-fold higher risk of still birth and is responsible for up to 35% of preventable low birth weight (Van et al., 2004), the prevalence of malaria is higher during pregnancy when compared with the non-pregnant state (Menedez et al., 1995). Garoua is the capital of the Northern Cameroon; there are very scarce reports and information on the incidence or prevalence of the disease or methods of control in the area. The study of malaria epidemiology in such area cannot be over emphasized.

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MATERIALS AND METHODS

Study area and study population

The study was conducted in Garoua, the North region of Cameroon. The region is bounded by the far north region to the north, the Adamawa region to the south, Nigeria to the west, Chad to the east, and Central African Republic to the southeast. The study was conducted from January to March 2007. Before commencement of the study, permission was sought from the government health. Meetings with pregnant women were held in each community (health care centers), to explain the objectives of the study and to obtain their consent. Structured questionnaires were used to collect information on socio demographic data, malaria mode of prevention and control, complaints, scientific knowledge about malaria, and laboratory examination.

Table 1. Distribution of malaria parasite among pregnant women living in different density in Garoua.

Location	No. examined	No. positive (%)
Low density	13	1 (1.03)
High density	163	77 (79.38)
Intermediate	64	19 (19.58)
Total	240	97

$\chi^2 = 5.99$, df = 2, $P < 0.05$.

Table 2. Distribution of malaria parasite among pregnant women using various control and preventive methods.

Preventive method	No. examined	No. positive (%)
None	101	40 (46.5)
Bed net	82	27 (32.9)
Drug	28	8 (28.6)
Insecticide	29	15 (51.7)
Total	240	97 (40.4)

$\chi^2 = 6.65$, df = 3, $P < 0.05$.

Table 3. Distribution of malaria among pregnant women using none or more than one methods of prevention and control.

Methods of prevention	No. examined	No. positive (%)
With control		
None	101	40 (43.9)
Bed nets + insecticide	5	0 (0.0)
With drug intake		
Bed nets + drug intake	45	4 (4.3)
Bed nets + insecticide	17	7 (7.6)
Insecticide + drug	72	40 (43.9)
Total	240	91

$\chi^2 = 0.74$ df = 4, $P < 0.05$.

Table 4. Distribution of malaria parasite among pregnant women of different occupation.

Occupation	No. examined	No. positive (%)
House wife (complete)	123	53 (54.63)
Trader/Business	76	28 (28.86)
Farmer	34	16 (16.49)
Student	5	0 (0. 0)
Civil service	2	0 (0.0)
Total	240	97

$\chi^2 = 0.087$, df = 4, $P < 0.05$.

Laboratory investigations

Thick and thin blood films were made on clean slides properly labelled for each subject. After patient's information has been recorded in the questionnaire, palm upwards, generally the third finger is selected and clean with cotton wool lightly soaked in the alcohol. With a sterile lancet, the ball of the finger is punctured using a quick rolling action. Pressure is applied to express blood about two to three drops on the slide (about 1 cm) for thick smear. The thick film was labelled with a marker at the edge of the slide. Slides were allowed to dry in a protected place from flies, dust and extreme heat in a slide space. Thick films slides were placed face downwards in a shallow tray on the slides and allowed to stain for 10 to 15 min. Afterward, the stain was gently flushed off the slides by adding drops of water, and the slides were placed in the film rack downwards to drain off water and dry (Monica, 1998).

Data collection and analysis

A computer program, Statistics Program of Social Sciences (SPSS) was used for data analysis. Frequency distribution tables, percentage prevalence and intensity of malaria infection were estimated using standard formulae; chi-square test was used as appropriate to test the possible effect of malaria parasite, as socio-demographic behaviour. 5% level of significance was used.

RESULTS

Table 1 describes the prevalence of malaria parasite among pregnant women in relation to population density in Garoua. Malarial infection rate was observed higher among pregnant women, living in high density area 79.38% (77/163). Women of low density area were relatively less infected 1.03% (1/13), especially as compared to pregnant women of intermediate area 19.58% (19/64). There was significant difference found among different density ($\chi^2 = 0.088$; $P < 0.05$). Preventive method against malaria infection was surveyed as shown in Table 2. Pregnant women using insecticide were less infected 16.66% (15/29) than those that were not using preventive method 44.44 (40/101) and bed net 30% (27/82). With regards to the use of prophylactic drugs, among the pregnant women, those that used prophylactic drugs had the lowest number of malarial infection with 8.88% (8/28). There was significant difference between the preventive methods used by pregnant women ($\chi^2 = 0.98$; $p < 005$). Table 3 shows the prevalence of malaria among pregnant women using none or more than one method of prevention and control. Malaria infection was found among women using insecticide and drug 40.81% (40/72), while those using bed nets, insecticide and drug together were not infected at all. Pregnant women that were using bed nets and drug intake were relatively less infected 4.08% (4/45) as compared to those with bed nets and insecticide 7.14% (7/17). Pregnant women that were not having preventive method were infected 47.9% (47/101). A significant difference was found between the methods of prevention ($\chi^2 = 0.036\%$; $P < 0.05$). The prevalence of malaria parasite among pregnant women in relation to their occupation is as shown in Table 4. The results show that farmer were highly infected 16.49% (16/34) than complete housewife 54.63% (53/123) and trader/business

Table 5. Distribution of malaria parasite among pregnant women at different stages of pregnancy.

Period of pregnancy (Months)	No. examined	No. positive (%)
0 – 3	30	13 (13.4)
3 – 6	132	48 (49.48)
6 – 9	78	36 (37.11)
Total	240	97

$\chi^2 = 0.088$, df = 2, $P < 0.05$.

28.86% (28/76). Civil servant and student had no infection. Table 5 shows prevalence of malarial in-fection among different stage of pregnancy. Women at their first trimester had 13.40% (13/30) infected, while those at their third and second trimester recorded 37.11% (36/78) and 49.48% (48/132), respectively. A significant difference was observed between malarial infection and pregnancy period ($\chi^2 = 5.99$; $P < 0.05$).

DISCUSSION

In low density settlement areas, the prevalence of malaria was relatively low among pregnant women. The infection is 1.03% conversely to those living in high density areas where the infection was high to 79.38%. However, given the large at-risk population in this malaria-endemic region of Garoua, low density settlement area is not a guaranty against malaria. High density settlement areas are characterised by the presence of dustbins, grasses, congestion, polluted gutter, stagnant waters, and dirty surroundings covered with weeds in the rivers that are suitable breeding sites for the presence of mosquitoes in the areas. This is consistent with the reports of Shr-jie et al. (2005) in Ouagadougou, Burkina Faso who found that higher prevalence rates of malaria occurred in areas where larvae breeding sites were semi-permanent. The study found that control and preventive methods com-monly used were bed nets, drug's intake (sulphadoxine pyrimethamine (sp)), intermittent preventive treatment (IPT) in pregnancy, and insecticide in homes among pregnant women. Those with no prevention and control method (44.44%) were more infected than those that were having bed net (30%) as method of control. This showed that bed net is a good mode of controlling mosquito's bites. This corroborate with Mouhamadou et al. (2006) that found that bet net efficacy help in reducing man-vector contact, malaria morbidity and mortality. The research proved that insecticide treated bed nets are amongst the effective tools at our disposal for reducing pregnant women mortality caused by malarial parasite. At dusk, some family will light flammable insecticide (coiled insecticide) that burns gradually during the night, repelling or killing mosquitoes. However, the smoke produced by such insecticide is sometime unbearable. The efficacy of insecticide has been proved in South Africa by Hargreaves et al. (2000) where dichlorodiphenyltrichloroethane (DTT) was used for house spraying to restore malaria control. Insecticide treated bed nets in pregnancy were associated with the first four months of pregnancies in Kenya where women receive as many as 45 to 230 infective bites on the average during 40 weeks gestation, and the result showed a significant reduction in parasitaemia during pregnancy, in the risk of low birth weight (D'alessandro and Olaye, 1997). The fact that the infection rate among those using bet nets and those using insecticide is almost equal explain that there may not be proper use of bed nets generally, they use the bed net after they have been exposed to mosquitoes outdoor. Series of trials in Africa have shown that proper use of mosquito net reduced malaria incidence among children from 63 to 14% in Ugandan (Frederick and Arinaitwe, 2003). Further evidence came from a recent study in a highly malarious area of Kenya. During the first four months of pregnancies, women who were protected by insecticide-treated bed nets at night gave birth to 25% fewer premature or small for gestational age babies than women who did not sleep under insecticide-treated bed nets; However, during the past decades, potentially more effective strategies for prevention and control of malaria in pregnancy have been developed and shown to have remarkable impact on the health of the mothers (Kuile et al., 2003). In Cameroon, the government via the public health centre protects pregnant women by giving bed nets to them (Plan Cameroon, 2005) with respect to WHO recommendations. The various reasons why some women were not sleeping under bed nets are: discomfort, sleepless, and difficulty in breathing. These days, antimalarial combination therapy is widely advocated and the use of artemesinin containing regimens is encouraged (WHO, 2000). In Thailand, when other treatments were failing, artesunate + mefloquine combination as first line treatment was successful and remains so many years later. In tropical Africa, WHO have pushed for artemether + lumefantrine and artesunate + amodiaquine combinations. Theoretically, artemisinin containing combinations might not only improve cure rates, but also reduce the speed at which resistance develop (Peter, 2004). In Cameroon, sulphadoxine-pyrimethamine (sp) is given at a therapeutic dose to pregnant women attending prenatal

centre. It is a single antimalarial dose with the best overall effectiveness for prevention of malaria in pregnancy in areas with high transmission and low resistance to sulphadoxine-pyrimethamine (WHO, 2000). The combine preventive methods and prevention in our research showed that people using bed nets and prophylactic drugs or insecticides were less infected than those using just bed nets. The lowest rate of infection was found among pregnant women using drugs as prophylactic treatment. However, 8.88% (8/28) were found infected. Interview with some women reveal that not all were having theirs drugs taken. They were flinging away the drugs just after they had left the medical center. Here again, reason like vomiting, discomfort, and dizziness was given. Though, in recommended dosage, drug should be generally well tolerated. Women are either stubborn or simply ignorant of the risk they are taking. The survey showed that infection was found at the three stages of pregnancy which was rather unfortunate, because of the adverse effect of malaria in pregnancy. Pregnant women at their third trimester were more infected than those at their first semester. The low infection rate recorded among pregnant women at their first trimester may be explained by the fact that some women are receptive and excited during the first trimester of their pregnancy. It also shows that proper and reinforced information could protect pregnant women in a better way and at different period of the pregnancy. Women at their third trimester are more infected than others; this may explain the fact that prevention against mosquitoes for many women is temporary and after some time, fail to protect themselves; this is clearly explain by the infection rate recorded by pregnant women at their second trimester, who are gradually neglecting malaria's prevention and increasing with infection rate.

The use of combined methods of control is good, because those that were using three control methods were not as infected as those that used only two prevention methods. This shows that the more the numbers of the combined preventive methods used the more the confidence about freedom from malaria. According to WHO (2001), new regiment should be incorporated into strategies aimed at controlling the spread of the disease, such as insecticides, spraying, and use of impregnated bed nets, sustained chemoprophylaxis and intermittent prophylactic treatment. Since none of these is used routinely in most parts of Africa, effective chemotherapy for malaria has a pivotal role in reducing morbidity and mortality (Schultz et al., 1994). Malaria infection was related to the occupation; civil servants and students were less infected than house wives who were majority Moslems and farmer in partial purdah, most of these pregnant women being illiterates are quite ignorant about malaria transmission and treatment. The considerable number of infection observed among traders/business women could be due to the fact that they are always travelling and do not have enough time to protect themselves against mosquitoes bite.

Conclusion

A combination of up to three methods of control/prevention is most active against acquisition of disease. Malaria is commonly found in pregnant women though many are asymptomatic, because of earlier contacts and development of immunity.

This study has evidenced the abundance of infection among pregnant women. Pregnant women at their third trimester were more infected than others. Pregnant women living in high density settlement area were more infected than those of low and intermediate areas. This study has shown that the infections were related to occupations and also to the poor knowledge of malaria mode of transmission. The infection is related to the level of education and the lack of improved continual education. Pregnant women are not participating in the fight against malaria. Bed nets are effective for reducing malaria transmission and mortality. The usage of bed nets, insecticide and therapeutic drugs gave considerable protection and should be promoted. These findings show that determining local endemicity and the rate of clinical malaria cases are urgently required in order to target control activities and avoid over-treatment with antimalarial.

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Full Length Research Paper

Feeding habits of culicine mosquitoes in the Cameroon lowland forests based on stable isotopes and blood meal analyses

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Mosquito blood feeding behavior is a very significant component of pathogen transmission and determinant of disease epidemiology. Yet, knowledge of foraging ecology of mosquitoes often depends on the presence of undigested blood in the mosquito mid gut. Approximately 36 h after feeding, the blood meal is sufficiently digested to make identification by molecular techniques difficult, leaving a very narrow window in which these methods can be utilized. Here, we investigated the feeding habits of wild caught culicine mosquitoes from four genera, *Aedes, Anopheles, Coquillettidia* and *Mansonia* of the lowland rainforests of Cameroon based on the isotopic ratios of nitrogen ($\delta^{15}N$), carbon ($\delta^{13}C$) and sulfur ($\delta^{34}S$). Results showed that unfed mosquitoes had a lower $\delta^{13}C$, $\delta^{15}N$ and middle $\delta^{34}S$ values than mosquitoes fed with $\delta^{13}C$ appearing to be the best element to differentiate between mosquito species that fed on different host species. Isotopic analyses show that the different mosquito genera may be separated based on their diets, suggesting that linking stable isotope-based assays and DNA analysis may be a powerful new tool to investigate mosquito feeding ecology and the dynamics of vector-borne pathogens.

Key words: Carbon ($\delta^{13}C$), nitrogen ($\delta^{15}N$), sulfur ($\delta^{34}S$), isotope, feeding habits, mosquitoes, polymerase chain reaction (PCR).

INTRODUCTION

Over the past decades, there has been an increase in the incidence of infectious diseases throughout the world, with serious implications for human and wildlife populations (Kilpatrick et al., 2006; Baker et al., 2012). Due to their diversity and abundance, demonstrated vector competence (Sardelis et al., 2002) and frequent infection in nature (Molaei et al., 2006; Burkett-Cadena et al., 2008a, b), mosquitoes are regarded as one of the most important vectors of disease.

The behavioral characteristics of mosquitoes in disease transmission differ vastly between different regions and species (Fontenille and Lochouarn, 1999; Kent and Norris, 2006), and can only be understood in a local context of vector-host interactions. An important aspect of these interactions is that of host preference, with each vector species feeding on a limited range of host species. Vector species that switch between hosts are particularly considered important in disease transmission because they have the potential to act as bridge vectors, by transferring pathogens from the reservoir to humans and/or domestic animals (Chandler et al., 1975; Rasgon, 2008). For example, West Nile Virus (WNV) is primarily an infection of birds, but can be transferred to humans or horses by mosquitoes that feed on both birds and mammals (Hamer et al., 2008). To accurately describe an

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arthropod-borne pathogen transmission cycle, it is critical to know which available host species are preferred by the corresponding vectors. Most studies evaluate the host-feeding patterns of mosquitoes by identifying the source of the vertebrate blood meals through sequencing portions of the cytochrome *b* gene of mitochondrial DNA. However, there is a very limited time period in which these methods can be utilized because these approaches only provide information on recent feeding events. After approximately 36 hours of feeding, the blood meal is sufficiently digested to make the identification by immunological or polymerase chain reaction (PCR)-based techniques particularly difficult, thus effective methods to assess feeding habits over longer periods of time are necessary.

Stable isotope techniques offer the opportunity to overcome some of these constraints by obtaining information on nutrients assimilated over extended periods of time (Rasgon, 2008). By comparing the isotopic signatures of a given organism or tissue, inferences on its feeding behavior could be done. Stable isotopes of carbon, nitrogen and sulfur have been widely used in ecological research (Wada et al., 1991; Fry, 2007). Stable carbon (δ13C); (δ15N) and nitrogen (δ34S) as indicated in ms are commonly used to extract information about the feeding habitat and carbon dietary sources (McCutchan et al., 2003; Michener and Lajtha, 2007; Yohannes et al., 2008); while nitrogen isotopes are often applied to evaluate the trophic position of a given species (Peterson and Fry, 1987). Based on the natural variation of the composition of stable sulfur isotope, measurement values can provide information on dietary protein sources and geographical origin (Richards et al., 2003). Also, because the rate of metabolism of different tissues determines the turnover of stable isotopes in tissues, it is possible to obtain dietary information on varying time-scales and extended period by sampling tissues with different turnover rates after feeding events (Tieszen et al., 1983; Rasgon, 2008).

The lowland forest areas in Cameroon are known to experience hyper-endemic transmission of *Plasmodium falciparum* mostly spread by the species, *Anopheles gambiae*, but little is known about the ecology of the different mosquito species in this region, and how vector species composition and their relative roles in transmission vary geographically (Akono et al., 2009). Very few studies on wild caught mosquitoes in Cameroon have been published (Rageau and Adam, 1952; Akono et al., 2009; Njabo et al., 2011), and very little information is available on their feeding habits (Snow and Boreham, 1978; Wanji et al., 2003).

In this study, we compared the feeding habits of wild caught blood-engorged mosquitoes and those with no visible blood meals (unfed mosquitoes or completely digested blood meal) that exhibit ornithophilic feeding habits, and characterize their feeding behavior by applying individual $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$. We also coupled our isotopic approach with PCR based molecular techniques and identified vertebrate host blood meals following extraction of DNA from the engorged mosquitoes and PCR amplification of the cytochrome *b* gene.

METHODOLOGY

Sampling sites and habitat characterization

Mosquito samples were collected in August, 2007 and May, 2008 at two lowland forest sites in Cameroon. Locations and dates of fieldwork for these forest sites were: Ndibi (N 2° 43' 50'', E 9° 52' 19''), August 21st to 26th, 2007 and May 2nd to 16th, 2008; Nkouak (N 3° 52', E 13° 18'), August 27th to 31st, 2007 and May 17th to 29th, 2008. Ndibi has an elevation of 500 meters above sea level and its habitat is characterized by disturbed lowland forest, and seasonally flooded swamp forest habitat and high human population (exacerbated by its close proximity to the city of Akonolinga), while Nkouak has less human population density and more forest cover and is the source of river Nyong. Rainfall patterns in these areas have been fairly stable for more than 40 years. There is one rainy season each year that lasts from November to April, followed by cool dry (April to August) and hot dry (August to November) seasons.

Mosquito collections

Two main trapping methods were used as described in Njabo et al. (2011). Briefly, six Center for Disease Control (CDC) Miniature Light Traps baited with CO_2 (John W. Hock, Gainesville, FL), and four modified bird-baited Ehrenberg lard cans (Downing and Crans, 1977). Three bird species Feral Pigeon (*Columba livia*), Yellow-whiskered Greenbul (*Andropadus latirostris*) and Greater Swamp Warbler (*Acrocephalus rufescens)* were used as baits in the lard cans.

Traps were set out each day for at least 12 h (06.00 pm to 06.00 am). Following each trapping period, the collection bags were removed from traps and the mosquitoes were transported to the field lab site where they were immobilized with chloroform and/or smoke. On the day of collection and immobilization, mosquitoes were sorted by sex and identified to species, with the aid of a stereomicroscope (× 90) and morphological keys (Hopkins et al., 1941; Service, 1990). The mosquitoes were then separated by gonotrophic condition (unfed, blood-fed, gravid) and enumerated. They were then sorted into four groups: Pigeon fed, Warbler fed, Greenbul fed and unfed (all mosquitoes with no visible blood meals in the abdomen are considered for the purpose of this manuscript as unfed). We used t-test to compare each fed group against the unfed mosquitoes (that is, binary comparisons of Pigeon fed, Warbler fed or Greenbul versus unfed were conducted separately). At both sites, sampling date and species, fed, unfed and gravid mosquitoes were pooled separately into groups of 5 to 20 specimens and placed in 95% alcohol and later stored at -20°C. Blood meals from blood-fed mosquitoes were subsequently tested by PCR, as described below, to determine the birds on which mosquitoes had fed.

Host blood meal identification

Within the pools of blood-fed mosquitoes, five individuals were randomly selected for PCR analyses. Total DNA was extracted from the abdominal contents of the blood-fed mosquitoes individually, using standard proteinase k digestion and phenol chloroform

purification. Isolated DNA from the mosquito blood meals served as DNA templates in subsequent PCR reactions. PCR primers (Forward 5'-3'GACTGTGACAAAATCCCNTTCCA, Reverse 5'-3' GGTCTTCATCTYHGGYTTACAAGAC) were based upon previously published multiple alignment of cytochrome *b* sequences of avian species (Ngo and Kramer, 2003). Amplified fragments from the blood-fed mosquitoes were sequenced and compared with sequences in GenBank for host-species identification as described in Molaei and Andreadis (2006) and Hellgren et al. (2008).

Stable isotope analyses

We analyzed stable carbon and nitrogen isotopes from representative whole mosquitoes using pooled samples within each group. We removed lipids from each homogenized sample, using a 2:1chloroform-methanol solution rinse for 24 h. Samples were rinsed using methanol solution and air-dried in a fume hood. Dried and powdered sub-samples, approximately 0.6 mg, were weighed into small tin cups to the nearest 0.001 mg, using a micro-analytical balance. Samples were then combusted in a Eurovector (Milan, Italy) elemental analyser (Limnological Institute, University of Konstanz, Germany). The resulting CO_2 and N_2 were separated by gas chromatography and admitted into the inlet of a Micromass (Manchester, UK) Isoprime isotope ratio mass spectrometer (IRMS) for determination of $^{13}C/^{12}C$, $^{15}N/^{14}N$ ratios. Measurements were reported in δ-notation ($\delta^{13}C$ and $\delta^{15}N$, respectively) where δ = 1000 × ($R_{sample}/R_{standard}$) - 1 ‰ relative to the Pee Dee Belemnite (PDB) for carbon and atmospheric N_2 for nitrogen in parts per thousand deviations (‰). Two sulfanilamide (Iso-prime internal standards) and two Casein were used as a laboratory standard for every 10 unknowns in sequence.

We also analyzed sulfur isotopes in mosquitoes. Tin capsules containing reference or sample were loaded into an automatic sampler from where they were dropped in sequence, into a furnace held at 1080°C and combusted in the presence of oxygen, raising the temperature in the region of the sample to ~ 1800°C. The reference material used for analysis was sulfanilamide calibrated and traceable to NBS-127 (barium sulphate, $\delta^{34}S$ = +20.3‰). Hundreds of replicate assays of internal laboratory standards indicate measurement errors (SD) of ± 0.05, 0.15 and 0.05‰ for $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$, respectively.

For each isotopic element separately, unfed versus fed mosquitoes of *Coquillettidia aurites* were compared using t-test. A second t-test was applied to compare elemental isotopic differences between blood-engorged *Coquillettidia pseudoconopas* and those with no visible blood meals. A one-way analysis of variance (ANOVA) followed by a Scheffe´ Post-hoc-test was used to compare differences in $\delta^{13}C$ and $\delta^{15}N$ between Warbler-fed, Greenbul-fed and unfed *Mansonia uniformis*

RESULTS

Five mosquito species belonging to four genera *Aedes* (*Aedes mcintoshi*), *Anopheles* (*Anopheles coustani*), *Coquillettidia* (*C. aurites and C. pseudoconopas*), and *Mansonia* (*M. uniformis*) were collected in the light traps while only three species (*C. aurites, C. pseudoconopas* and *M. uniformis*) were collected in the lard cans. All the species collected from the CDC light traps were unfed (had no visible blood meals) while all species from the bird-baited lard cans were blood-engorged.

Host blood meal identification

To confirm the applicability of the blood meal-specific PCR to Cameroon lowland forest mosquitoes and their avian hosts, the assay was tested for its ability to amplify cytochrome *b* sequences from several native Cameroon lowland forest bird species (results published elsewhere (Chasar et al., 2009; Njabo et al., 2011). The specific amplification conditions were found to support the amplification of detectable PCR products from all the bird species tested. The PCR diagnostic successfully identified all blood meals from engorged mosquitoes collected from the baited lard cans. No other species were identified and none of the mosquitoes fed on multiple hosts, indicating that the bias towards bird feeding was not indicative of a specific host preference for birds but a reflection of relative host availability. All unfed mosquitoes tested were negative.

Mosquito stable isotope analyses

The $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ values of unfed mosquitoes and fed mosquitoes are shown in Table 1. In general, unfed mosquitoes have relatively lower $\delta^{13}C$ and $\delta^{15}N$ compared to the blood-engorged mosquitoes. Mean $\delta^{13}C$ values from unfed mosquitoes ranged from ca.-20.0 to -34.5‰, while $\delta^{15}N$ ranged from 3.1 to 4.8‰, and $\delta^{34}S$ ranged from 6.4 to 10‰. Mean $\delta^{13}C$ values from fed mosquitoes ranged from -19.8 to -24.5‰, while $\delta^{15}N$ ranged from 4.3 to 7.1‰, and $\delta^{34}S$ ranged from 5.5 to 8‰.

The mean ± SE of $\delta^{13}C$ and $\delta^{34}S$ of each mosquito species are given in Figures 1 and 2, and Table 1 separately. Fed and unfed *C. aurites* showed a significantly different $\delta^{13}C$ (t-test, p = 0.02) signature (Figure 1). However, there was no difference between fed and unfed $\delta^{34}S$ (Figure 2) or $\delta^{15}N$ (Figure 1). Blood-engorged *C. pseudoconopas* and those with no visible blood meals showed significant difference at both $\delta^{13}C$ (t-test, $p < 0.0001$) and $\delta^{34}S$ ($p = 0.01$, equal variance not assumed).

ANOVA revealed that there was a significant diet effect on $\delta^{13}C$ and $\delta^{15}N$ values of *M. uniformis* ($\delta^{13}C$: $F_{15,98} = -7.19$, $p < 0.001$; $\delta^{15}N$: $F_{12,97} = -3.65$, $p < 0.001$). Post-hoc analysis of $\delta^{13}C$ for Warbler-fed and Greenbul-fed *M. uniformis* species indicated no significant difference ($p > 0.05$), and thus we pooled these data in further analyses and compared unfed and fed *M. uniformis* using t-test. On the contrary, *M. uniformis* that fed on Warbler were significantly different in $\delta^{15}N$ (t-test: t =14.44, p = 0.003 equal variance not assumed due to unbalanced sample size) than those that fed on Greenbul (Figure 1 and Table 1).

Bi-directional error bar graph (Figure 1) for $\delta^{13}C$ and $\delta^{15}N$, for fed (grouped together in a rectangle frame) and unfed groups show that unfed *A. mcintoshi* survives on

Table 1. Mean ± SE (n) values of nitrogen, carbon and sulfur Isotopes of unfed and fed mosquitoes.

Species	Diet	$\delta^{15}N$ (‰)	t-test	$\delta^{13}C$ (‰)	t-test	$\delta^{34}S$ (‰)	t-test
C. aurites	Unfed	4.5±0.31(8)	t=0.23	-30.4±3.5 (8)	t=-2.52	6.4±0.46 (3)	t=0.80
*C. aurites**	Pigeon	4.3±0.78 (9)	p=0.82	-21.8±2.47 (9)	p=0.02*	5.9±0.59 (9)	p=0.44
C. pseudoconopas	Unfed	4.8±0.37 (6)	t=-0.90	-34.7±1.07 (6)	t=-0.77	6.8±0.34 (6)	t=0.33
*C. pseudoconopas**	Pigeon	5.2±0.11 (3)	p<0.0001*	-19.8±1.05 (3)	p=0.46	5.4±0.28 (3)	p=0.01*
M. uniformis	Unfed	3.1±0.23 (90)	t=-14.25	-29.9±0.48 (90)	t=-7.52	8.4±0.36 (3)	t=-1.29
M. uniformis[1]	Greenbul	7.1±0.51 (6)	p<0.0001*	-24.5±1.80 (6)	p<0.0001*	8.9±0.36 (3)	p=0.27
M. uniformis[2]	Warbler	5.8±0.23 (3)	t =-11.21	-23.7±1.11 (3)	p<0.0001*	8.9±0.36 (3)	p=0.27
A. Mcintoshi	Unfed	3.9±0.34 (17)	p<0.0001*	-20.0±0.64 (17)	p<0.0001*	8.0±0.47 (3)	p<0.0001*
A. coustani	Unfed	4.0±0.63 (3)	p<0.0001*	-26.8±0.52 (3)	p<0.0001*	10.3±0.09 (3)	p<0.0001*

Diet: *Feral Pigeon, [1]Yellow-whiskered Greenbul and [2]Greater Swamp Warbler fed mosquitoes in the lowland forests of Cameroon. Results of the Student's t-test are given for each comparison. *Statistically significant differences (p<0.05). $\delta^{13}C$ (‰) in unfed *M. uniformis* was compared using pooled Greenbul and Warbler fed *M. uniformis*. Sample sizes for $\delta^{34}S$ are lower than for $\delta^{15}N$ or $\delta^{13}C$ due to missing samples.

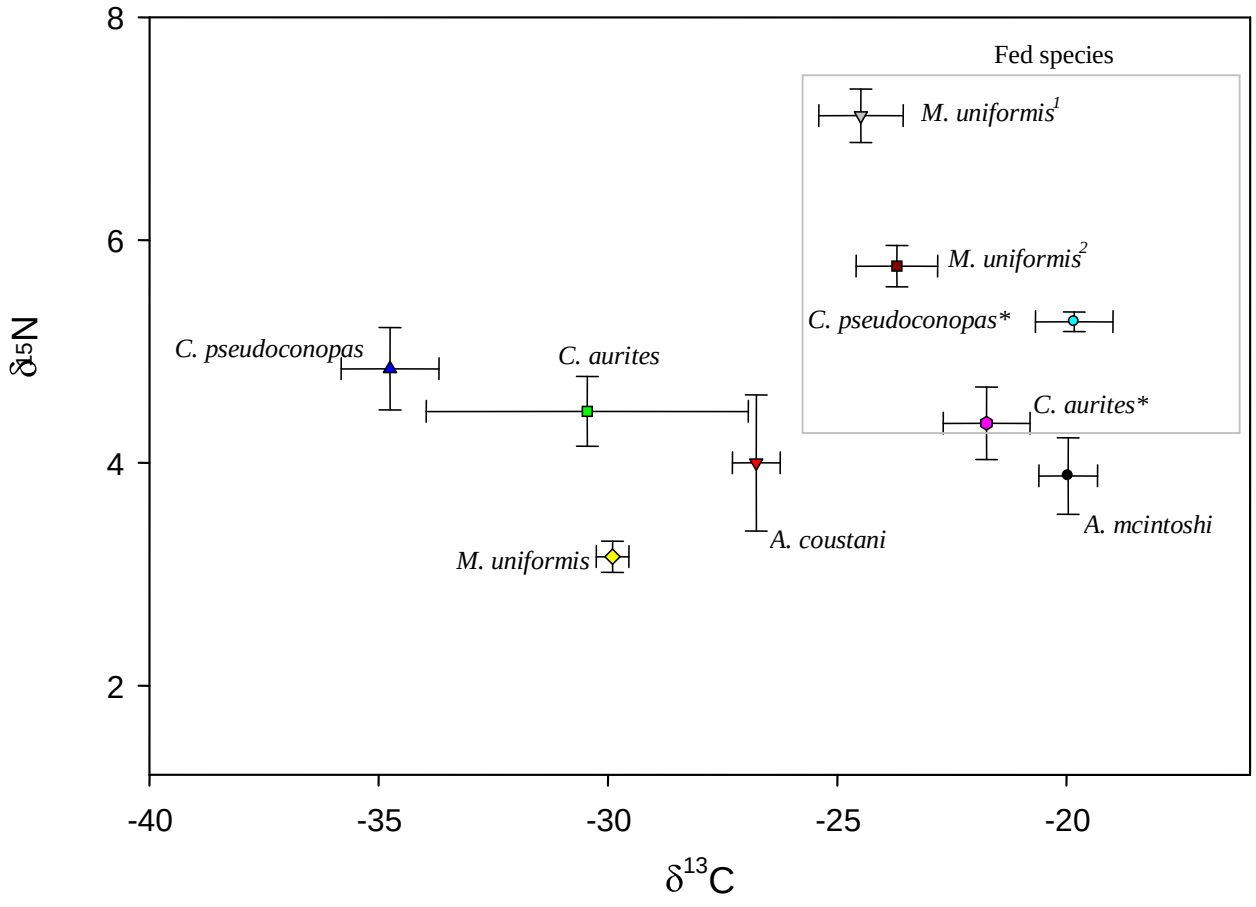

Figure 1. Bi-directional mean (± SE) values of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) of unfed and blood-engorged mosquitoes in the lowland forests of Cameroon. Mosquitoes were fed with *Feral Pigeon, [1]Yellow-whiskered Greenbul and [2]Greater Swamp Warbler.

coustani, C. aurites and *C. pseudoconopas*, respectively. These values for *A. mcintoshi* are very similar to those of *Coquillettidia* species that fed on pigeons. As shown in Figure 1, the highest $\delta^{34}S$ in these study species was recorded for *A. coustani*. This species also showed an intermediate $\delta^{13}C$ values (ca. -27‰) and $\delta^{15}N$ (ca. 4‰), indicating a unique sulfur dietary sources in this species Interestingly, *M. uniformis* which fed on warblers has a comparable $\delta^{34}S$ (ca. 9‰) to that of *A. coustani* (ca. 9‰) signature. This implies the two mosquito species rely on

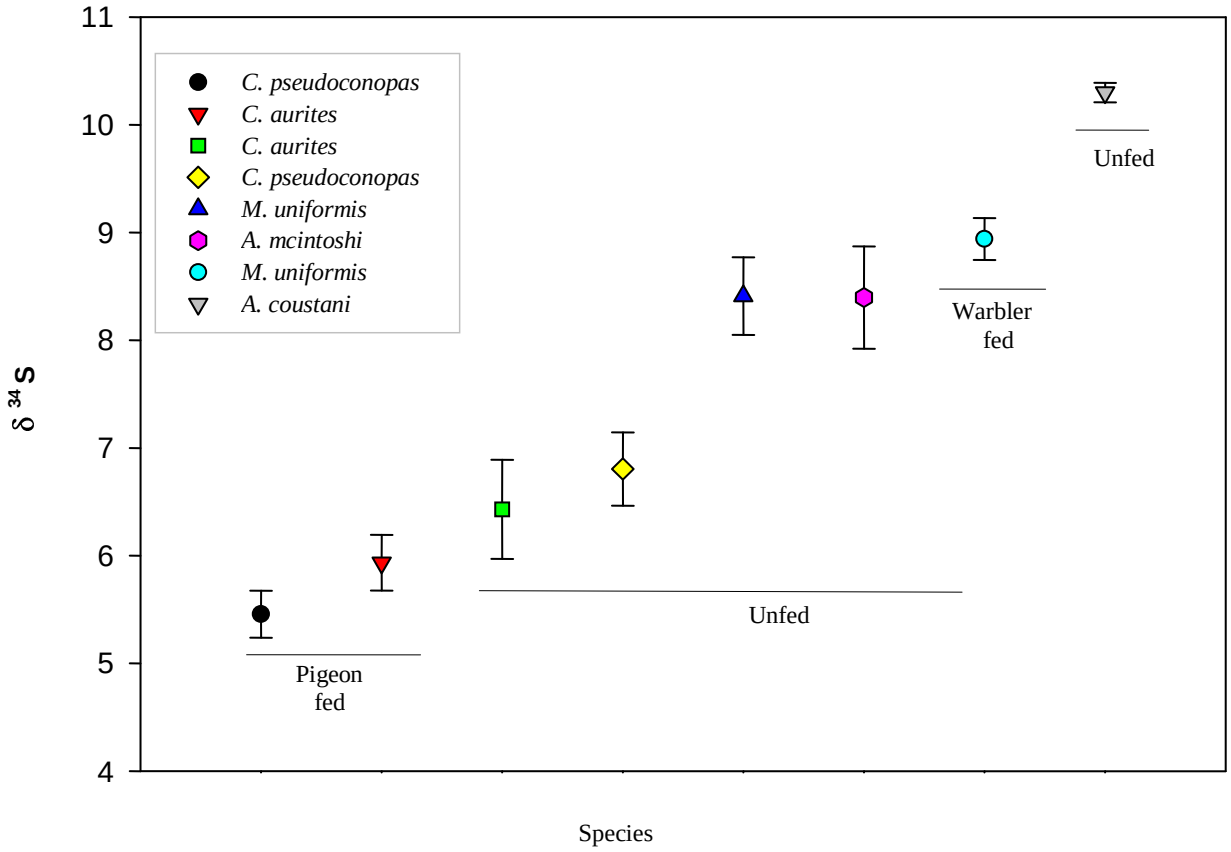

Figure 2. Mean (± SE) values of carbon ($\delta^{34}S$) for unfed and fed (Pigeon and Warbler fed) mosquitoes collected from the lowland forests of Cameroon.

similar sulfur dietary source.

DISCUSSION

In this study, four mosquito genera, *Aedes, Anopheles, Coquillettidia* and *Mansonia* and three different host types, were examined for triple isotopic elements: $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$.

Stable carbon isotopes

Our results suggest that the five mosquito species studied exhibit a wide-range of $\delta^{13}C$ signature spanning from ca -49 to -15‰, indicating a variety of host-carbon source in the region. These results support the findings of Hassan et al. (2003) who showed that ornithophilic mosquitoes typically feed significantly more or less on available bird species than predicted based on biomass, surface area, or relative abundance.

Although these mosquitoes seem to potentially cover a rather wider carbon source, each species shows a more specific and distinctive isotopic niche-width. For instance, *A. mcintoshi* apparently feeds on a host with a relatively higher stable carbon isotope value. Using mean $\delta^{13}C$ values reported for C_4 (-13‰) and C_3 (-27‰) plants (Michener and Lajtha, 2007) as endpoint tissue, $\delta^{13}C$ of *A. mcintoshi* departs from a dietary source based on C_3 plant and supports a C_4 dominated carbon source incorporated in its food chain. Relatively higher $\delta^{13}C$ values, as those reported for this species, can also be linked to C_3 plants of drier areas and xeric condition in response to water-use efficiency (Ehleringer and Cooper, 1988).

The $\delta^{13}C$ values of *A. mcintoshi* also complement those of *Coquillettidia* spp. that fed on pigeons, indicating that *A. mcintoshi* may also feed on pigeons or on other avian/vertebrate hosts with similar food habits. Pigeons are known to forage mainly in agricultural landscapes, as these areas represent an important and well exploited source of food (Hetmanski et al., 2005). There were no significant differences in isotopic values in $\delta^{13}C$ ($p > 0.05$) for Warbler-fed and Greenbul-fed *M. uniformis*. Although we observed slight differences in *M. uniformis* that fed on Warblers to those that fed on Greenbul (of $\delta^{13}C$ and also $\delta^{15}N$), the wide-range of isotopic signatures from both fed and unfed individuals (for example, mean $\delta^{13}C$ range: ca.

-24 to -30‰ of this species) suggest that *M. uniformis* seems to be a generalist that feeds on available host species of different isotopic values.

Indeed, *Mansonia* mosquitoes are major vectors of filariasis caused by *Brugia malayi* filarial nematodes and feed on a wide range of vertebrate hosts in nature (Laurence, 1960; Phumee et al., 2011). A larger range of $\delta^{13}C$ was also observed for unfed *C. aurites*. -21 to -48‰, implying the availability of multiple host species or hosts that rely on wider isotope biomes. Analysis of additional isotopes elements such as deuterium or oxygen may improve the resolution of nutrient sources in blood meal identification, especially in situations of many potential host species.

Stable nitrogen isotopes

The $\delta^{15}N$ signatures in consumer tissues are primarily used to assess trophic position in food webs. It has been shown that $\delta^{15}N$ values can be associated with plant and animal tissues grown in relatively low rainfall locations and arid environmental conditions (Ambrose, 1991; van der Merwe et al., 1993; Sealy et al., 1995). Our study illustrates that almost all unfed mosquito species exhibited similar nitrogen isotope signature. However, Greenbul-fed *M. uniformis* showed a much more enriched (up to Δ + 4‰) $\delta^{15}N$ than unfed conspecific species indicating a higher trophic position than the other mosquito species. We also measured a slight difference in $\delta^{15}N$ of *M. uniformis* that fed on Warblers and those fed on Greenbul.

Stable sulfur isotopes

A. coustani seems to depend on a higher sulfur isotopic niche. *M. uniformis* which fed on warblers has a clearly separated nitrogen (Figure 1) and sulfur isotope (Figure 2) compared to Pigeon-fed species. The similar $\delta^{34}S$ in Warbler-fed and unfed *M. uniformis,* as well as to unfed *A. mcintoshi* imply that these two mosquito species might feed on similar hosts. By implication, while others occupy a unique specific niche, others do share host sources and rely on similar nutrient sources. In summary, our results illustrate the advantages of stable isotope analyses for the study of mosquito host feeding and niche segregation analyses. Using both fed and unfed mosquitoes collected in the field, we showed that the stable isotopes-based assays could play an essential role as tracers when applied on specimens unsuitable for PCR, such as gravid individuals or mosquitoes with digested or no observable blood meal to reconstruct the history of previous feeding events and dietary sources.

While stable isotope profiling is more sensitive in tracing element and nutrient sources, it is less specific in identifying digested blood meals to specific species as PCR techniques are. Thus the best results are obtained when both methods, stable isotope profiling and PCR, are used in concert. PCR may be used on fresh blood meals to gain a recent snapshot of current feeding habits, while stable isotope analysis may be used to reconstruct dietary history, trophic status and niche width of vectors over the longer time frames.

In general, field situations are likely to be more complicated, with multiple potential hosts present in the environment. Additional analyses of stable isotope ratios through more extensive sampling of potential hosts may provide further insights into seasonal changes in host preference and individual species' dietary strategies. To fully get the complete picture of field situations therefore, initial stable isotope profiling will have to be performed against all potential hosts in the study area, and temporal-spatial variation in stable isotope profiles within populations and within individual mosquitoes taken into account.

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UPCOMING CONFERENCES

24th International Conference of the World Association for the Advancement of Veterinary Parasitology, Perth, Australia, 25 Aug 2013

XXI Latin American Congress of Parasitology, Guayaquil, Ecuador, 6 Oct 2013

Conferences and Advert

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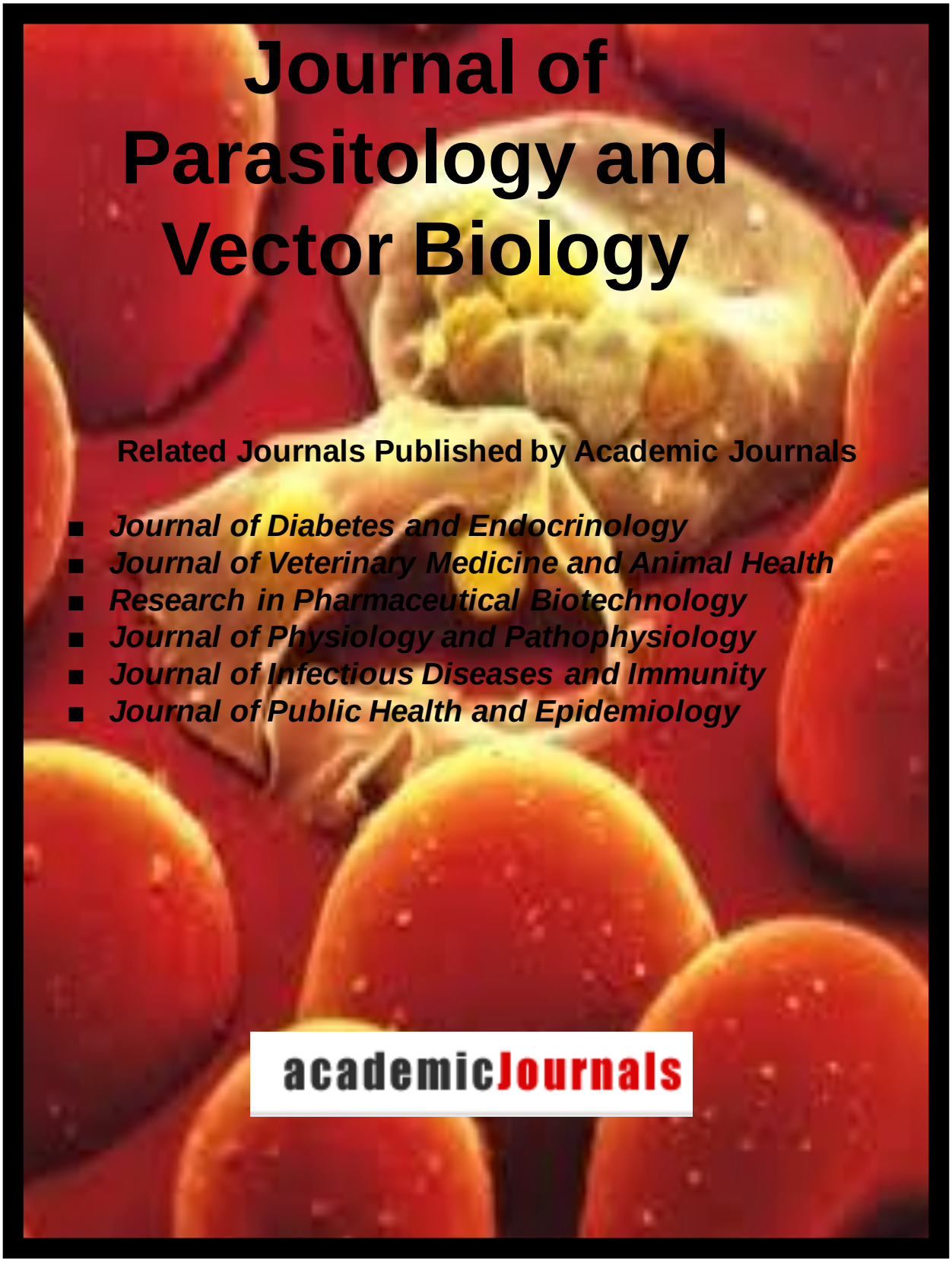
24th International Conference of the World Association for the Advancement of Veterinary Parasitology, Perth, Australia, 25 Aug 2013

September 2013
5th European Congress of Virology, Lyon, France, 11 Sep 2013

October 2013
XXI Latin American Congress of Parasitology, Guayaquil, Ecuador, 6 Oct 2013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